Bioactive mesopore-glass microspheres with controllable protein-delivery properties by biomimetic surface modification

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Abstract

Microsphere systems with the ideal properties for bone regeneration need to be bioactive, and at the same time possess the capacity for controlled protein/drug-delivery; however, the current crop of microsphere system fails to fulfill these properties. The aim of this study was to develop a novel protein-delivery system of bioactive mesoporous glass (MBG) microspheres by a biomimetic method through controlling the density of apatite on the surface of microspheres, for potential bone tissue regeneration. MBG microspheres were prepared by using the method of alginate cross-linking with Ca^{2+} ions. The cellular bioactivity of MBG microspheres was evaluated by investigating the proliferation and attachment of bone marrow stromal cell (BMSC). The loading efficiency and release kinetics of bovine serum albumin (BSA) on MBG microspheres were investigated after coprecipitating with biomimetic apatite in simulated body fluids (SBF). The results showed that MBG microspheres supported BMSC attachment and the Si containing ionic products from MBG microspheres stimulated BMSCs proliferation. The density of apatite on MBG microspheres increased with the length of soaking time in SBF. BSA-loading efficiency of MBG was significantly enhanced by co-precipitating with apatite. Furthermore, the loading efficiency and release kinetics of BSA could be controlled by controlling the density of apatite formed on MBG microspheres. Our results suggest that MBG microspheres are a promising protein-delivery system as a filling material for bone defect healing and regeneration.

Key words: Bioactive mesopore-glass microspheres; Controllable protein delivery; Apatite; Cell proliferation

INTRODUCTION

The use of bioactive microspheres as protein/drug carriers and bone filling materials has received

much attention in recent years.[1,2] Compared to traditional macroporous block scaffolds, the main advantage of microspheres is that they not only possess better drug-delivering properties, but also the ability to fill bone defects of irregular and complex shapes and sizes. The space between the microspheres is crucial for effective and functional bone regeneration [3-5] as they allow for both bone and vascular ingrowths. However, for microspheres to be applied as bone fillers in bone-tissue regeneration there are three major issues that needs to be resolved: (i) controllability of drug-release; (ii) bioactivity; and (iii) degradability. Currently available microspheres are made of ceramics, polymers or their composites. Polymers microspheres, such as poly(lactic-co-glycolic acid) (PLGA) and poly(hydroxybutyrate-polyhydroxyvalerate) (PHBV), are resorbable but their bioactivity is less than optimal. [2,6] On the other hand, ceramic microspheres, such as hydroxyaptite (HAp) ceramics with large size (several hundred micrometer), are bioactive, but lack controllable protein/drug ability and adequate degradation.[1,7-10] Although nano-sized spheres have been developed for drug delivery, their small sizes compromise their use in cell-based therapy for bone tissue engineering. Their nanometer sizes result in an inadequate space for tissue and vascular ingrowth and they are also too small to carry bone forming cells. Therefore, it is necessary to develop a new class of larger sized bioactive microspheres (hundreds of micrometer) with controlled protein/drug-delivery properties and improved bioactivity and degradation rates.

Bioactive glass is a typical bioactive inorganic material in that it can bond with host bone tissue by forming a biologically active bone-like apatite layer after implantation.[11] Compared to other bioactive ceramics, such as HAp and β-tricalcium phosphate (β-TCP), bioactive glass possesses greatly improved bioactivity and degradation properties.[11] A new class of bioactive glass was recently developed with a highly ordered mesopore channel structure with pore sizes ranging from 2 to 50 nm, referred to as bioactive mesoporous glass (MBG).[12,13] A significant feature of MBG is

that it possesses a significantly improved specific surface area and pore volume, which gives it superior bioactivity compared to non-mesoporous bioactive glass (NBG).[12,14] Recently, we have shown that the incorporation of MBG powders into PLGA polymers significantly enhanced the bioactivity, degradation rate and drug-delivery properties of PLGA.[15] In addition, studies by other investigators have shown that MBG powders possess significantly improved drug loading ability as a result of their uniquely ordered channels and high specific surface area, when compared to NBG powders.[16,17] These findings suggest that MBG micropsheres have far greater potential for bioactive bone regeneration and drug delivery than the more traditional materials. However, to the best of our knowledge, most of the present studies of MBGs are focused on MBG powders, which severely limit their further application, in spite of their excellent bioactivity and degradation. It is therefore reasonable to hypothesize that MBG microsphere with larger size and controllable protein delivery will be a more satisfactory bone filling material.

Previous studies have shown that biologically active molecules, such as growth factors, can be co-precipitated with CaP crystals on the surface of metal implants without compromising the bioactivity of osteogenic agents due to the mild conditions employed during the coating process [18]. It has been reported that the CaP coatings incorporated with bioactive molecules can substantially improve the bioactivity and enhance the osteogenic activity of the metal implant.[18-20] A recent study incorporated proteins into the CaP coatings on polymers to enhance the binding ability of the proteins.[21] However, few, if any, studies have investigated the controlled loading and release of proteins/drugs from biomimetic apatite. Since it is known that MBG has excellent apatite-forming ability in simulated body fluid (SBF), our hypothesis was that by controlling the soaking time of MBG in SBF, the density of apatite on the MBG microspheres could be controlled, thereby controlling both protein loading and release. The aim of this study was to prepare and characterize

MBG microspheres, and further to control the density of apatite on the microspheres and hence protein loading and release for potential bone tissue regeneration application.

MATERIALS AND METHODS

Preparation and characterization of MBG microspheres

MBG powders (molar ratio: Si/Ca/P = 80/15/5) were synthesized according to our previous publication.[22] In a typical synthesis, 4.0 g of P123, 6.7 g of tetraethyl orthosilicate (TEOS, 98%, Acros), 1.4 g of $Ca(NO_3)_2.4H_2O$, 0.73 g of triethyl phosphate (TEP,99.8%, Sigma–Aldrich) and 1.0 g of 0.5 M HCl were dissolved in 60 g of ethanol (Si/Ca/P¼ 80:15:5, molar ratio) and stirred at room temperature for 1 day. The resulting sol was introduced into a Petri dish for an evaporation-induced self-assembly process, and then the dry gel was calcined at 700 ºC for 5 h to obtain MBG powders. The obtained MBG powders were ground and sieved to 230 meshes for the preparation of MBG microspheres by using a method of alginate crosslinking with $CaCl_2$ solutions. Typically, 3.125 gram of MBG powder was added into 40 mL water solution and stirred for 30 min to form slurry. Alginate powder was then dissolved in water to form the alginate solution with a concentration 3% (w/v). The alginate solution was added to MBG slurry and stirred for 1 hr and ultra-sonicating for 5 min to form homogenous mixtures. The mixture was extruded drop-wise into a 0.1 M $CaCl_2$ crosslinking solution through a 0.80 mm hypodermic needle, forming spherical particles, which were hardened by 30 min incubation in the crosslinking solution. The wet microspheres were filtered, dried at 50 ºC and calcined at 680 ºC for 3 hr with a heating rate of 1 ºC/min to obtain the MBG microspheres. The phase composition, surface morphology, element composition and inner microstructure of the calcined MBG microspheres were analyzed by wide-angle X-ray diffraction (XRD), small-angle XRD, Transmission electron microscopy (TEM), optical microscopy, scanning

electron microscopy (SEM) and energy-dispersive spectrometer (EDS). The surface area, the pore size distribution and the pore volume were tested using N_2 adsorption-desorption isotherms. The open porosity of MBG microspheres was tested using Archimedean method.

The effect of ionic products from MBG microspheres on BMSC proliferation

The bone marrow samples ($n=5$) for isolation of human bone mesenchymal stem cells (BMSCs) were obtained Prince Charles Hospital, Brisbane. Ethics clearance for this project was approved by the Human Ethics Committee at Queensland University of Technology. BMSC culture was carried out according to our previous publications.[23] The extracts of MBG microspheres were diluted in culture medium according to International Standard Organization (ISO/EN 10993-5) [24] by adding MBG microspheres to serum free α-MEM culture medium at a ratio of 200 mg/mL (MBG to medium). After incubating at 37ºC for 24 hr, the mixture was filtered and the supernatant was collected. Serial dilutions of extracts (100, 50, 25, 12.5 and 6.25 mg/ml) were prepared using serum-free α-MEM medium (without L glutamine or ascorbic acid). The diluted extracts were filtered using 0.22 μm filter and used in subsequent BMSC cell culture experiments. The ionic concentration of the diluted extracts was measured by atomic emission spectrometer (Table 1). BMSCs were seeded at a density of 3×10^3 cells/well into 96-well plate and incubated for 24 hr. The culture medium was then removed and replaced by 50 μL of α-MEM medium supplemented with 10% FCS and 50 μL of diluted extracts. Culture medium supplemented with 10% FCS without addition of diluted extracts was used as a blank control (Blank), and 50 μL solution of 0.2% Triton X-100 and 50μL of α-MEM medium supplemented with 10% FCS was used as a negative control (Ctr-). Cells were then incubated at 37ºC in 5% CO_2 for 1, 3 and 7 days. At these time points 100 μL of 0.5 mg/mL MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution

was added in each well and incubated for 4 hr at 37ºC. The reaction was terminated by the addition of dimethyl sulfoxide. The absorbance of the formazan was read at 495 nm using an Enzyme-linked immunosorbent assay (ELISA) plate reader. This assay is based upon the conversion of MTS to formazen. Results were expressed as absorbance reading from each well minus the optical density value of blank wells.

BMSCs attachment on MBG microspheres

MBG microspheres were transferred into 96 wells. After pre-wetted with serum free culture medium overnight, 100 μL cell suspensions containing 5×10^5 BMSCs were seeded into each well. After 72 hr the cells were fixed in 4% paraformaldehyde for 20 min and washed in PBS, permeabilized in 0.5% Triton for 15 min, and washed again in PBS. After washing, filters were incubated for 1 hr at room temperature with Alexa Fluor™ 568 phalloidin (1:50 dilution; Invitrogen A12380) diluted in 0.1% PBS/BSA, washed with PBS at RT for 3 x 5 min, then labeled with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Sigma-Aldrich, St. Louis, MO) for 30min. DAPI is a nuclear fluorescent stain. Samples were washed with PBS and examined with a Leica SP5 confocal microscope (Mannheim Germany).

Preparation and characterization of BSA-loaded MBG microspheres with apatite layer

Simulated body fluids (SBF) were prepared according to Kokubo.[25] Then, 100 mg of BSA (Sigma) was dissolved in 50 mL SBF solution. 0.075 g of MBG microspheres were immersed in the BSA/SBF solution and kept under static conditions at 37 ºC for 1, 3 and 7 days. The bovine serum albumin (BSA) is commonly used in several studies to simulate human albumin, due to the structure homology of two proteins.[26] The microspheres were labeled MBG-SBF-1d, MBG-SBF-3d

and MBG-SBF-7d according to the length of time soaking in the SBF solution. After soaking in SBF the BSA-loaded microspheres were dried at 60ºC for 24 hr. Apatite formed on the surface of MBG microspheres was analyzed using SEM, EDS, Wide-angle XRD and Fourier transform infrared spectroscopy (FTIR). For controls, BSA-loaded MBG microspheres without apatite layer were prepared by soaking 0.075g of MBG microspheres in 50mL BSA water solution (2mg/ml) for 24 hr.

BSA loading and release kinetics from MBG microspheres with apatite layer

To determine the loading ability of BSA on MBG, MBG-SBF-1d, MBG-SBF-3d and MBG-SBF-7d microspheres, the weight loss profiles of the four dried BSA-loaded MBG microspheres species were analyzed by thermogravimetric analysis (TGA) from room temperature to 800 ºC. BSA loading efficiency (LE) was calculated using the equation: LE = (mass of BSA/mass of MBG microspheres) * 100%, according to weight loss profile at the range of 200-800ºC.

For evaluation of the effect of apatite layer on the BSA release, 0.045g of BSA-loaded MBG microspheres were placed into 1 mL PBS (pH = 7.4) at 37 ºC for 1, 3, 6h, 1, 3, 7, 14, 21 and 28 days. At each time point, 1 mL PBS solution was taken out to test the concentration of released BSA by UV/VIS at the wavelength of 280nm and 1 mL fresh PBS solution was added to the plastic tubes containing the microspheres. Three samples for each kind of microspheres were taken for mean and standard deviation calculation.

Statistical analysis

The data was expressed as means ± standard deviation (SD) for all experiments and were analyzed using one-way ANOVA with a post hoc test in ORIGIN statistic software, where p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Characterization of MBG microspheres

The resulting MBG microspheres were approximately 1 mm in diameter with uniform size distribution (Fig. 1a and b), and composed of MBG microparticles with a loose microstructure (Fig. 1c). Both the surface and in the interior of the microspheres contain numerous micropores (Fig. 1c and d). EDS analysis of the microspheres revealed an abundance of Ca, Si and P elements (Fig. 1c). Small angle XRD results showed that the microspheres had an apparent differential peak at $2\theta = 1.25^{o}$ (Fig. 2a), which indicates that the MBG has mesopore walls with a hexagonal structure. The open porosity of the obtained MBG microspheres is about 37%. TEM analysis showed that MBG microspheres possessed a highly ordered nano-mesopore channel structure and that the pore diameter was approximately 5 nm across (Fig. 2b). The results for N_2 adsorption-desorption analysis have shown the type of IV isotherm, a typical characteristic of a mesoporous structure (Fig. 2c). MBG microspheres presented a relative narrow pore size distribution, and the peak pore size is around 5nm (Fig. 2c), which is consistent with the result obtained by TEM analysis. The pore size distribution is mainly in the range of 3nm to 11nm, and the BET surface area of MBG is 336m2/g. As after microspheres were sintered, we then crashed microspheres to be powders, and then we found that the size of nanopores in the powders is 5nm. Therefore, the nanopore is within the microspheres. These results confirmed that large-sized MBG microspheres are obtainable by the described method, and that alginate crosslinking with $CaCl_2$ solution, followed by heat treatment does not alter the highly ordered channel structure of MBG. This indicates that the microspheres are capable of maintaining the inherent characteristics of mesopore materials, such as high specific surface area and high bioactivity.[16,22] A further advantage of this production method is that the MBG

microspheres have a relatively large size of 1 mm. This provides sufficient surface area for bone cell attachment and growth as well as large interstitial space (room) between the microspheres for both tissue and vascular ingrowth, both essential properties for effective and functional bone regeneration.[3,4] Furthermore, the loose structure and high micropore content inside the MBG microspheres provide adequate space for drug/protein loading.

BMSCs response to MBG microspheres

The ionic products of MBG microspheres significantly stimulated BMSC proliferation compared to the blank controls after 3 days in culture (Fig. 3). Calcium and silicon ions can stimulate osteoblast proliferation, differentiation and osteogenic gene expression [27-30] and our data showed that Si ion concentrations in MBG extracts were significantly higher than those of the controls, whereas Ca ion concentrations were similar between MBG extract and the controls (Table 1). It is therefore plausible that Si ions from the MBG microspheres are the main factor responsible for stimulating cell proliferation. However, this stimulatory effect depends on a certain concentration range. In a specific concentration range of Si ions, although all Si containing MBG extracts have enhanced cell proliferation compared to blank control without Si ions, the cell proliferation for these Si containing MBG extracts has no obvious difference. According to published assessment criteria; this stimulatory effect is regarded as one of the evaluation criterions for the bioactivity of biomaterials.[27,31] Our results indicate that the MBG microspheres have the potential to stimulate bone tissue growth by releasing bioactive Si ions and are therefore promising materials for bone repair applications. Indeed, cell experiments demonstrated that MBG microspheres supported BMSC attachment and growth (Fig. 4), indicating that the microspheres are suitable as cell carrier for potential tissue engineering applications.

Characterization of apatite formation on MBG microspheres as a function of soaking time

Controllable apatite deposition on the MBG microspheres was achieved by soaking the spheres in SBF for various lengths of time. SEM analysis showed that apatite particles deposited on the microsphere surface increased as a function of soaking time (Fig. 5) and the spheres appeared to maintain their rounded morphology (Fig. 5a, b and c). At day 1, only a limited number of apatite particles were seen on the microsphere surface (Fig. 5d). The ratio of Ca/P was 2.52 (Fig. 5g), which was less than that prior to soaking at 3.06. A loose apatite layer was apparent after 3 days of soaking and there were discernible peaks of Ca and P elements (Fig. 5e and h). After 7 days, a dense apatite layer, composed of apatite particles with an obvious crystalline morphology, had formed. Ca and P elements were found only in the deposited apatite (Fig. 5i). Wide angle XRD patterns showed that with increased soaking time, the intensity of apatite characteristic peaks (standard card No JCPD:24-0033) increased and that wollastonite ($CaSiO_3$) peaks decreased (Fig. 6). By day 7, only apatite peaks were seen and the wollastonite peaks had entirely disappeared (Fig. 6). FTIR analysis showed that prior to soaking in SBF the MBG microspheres had a overlap peak of PO_4^{3-} at the wave number of 1030 cm^{-1} and Si-O at the wave number of 980 cm^{-1} (Fig. 7). Wave number 566 cm^{-1} revealed a small PO_4^{3-} peak from microspheres that had soaked in SBF for 1 and 3 days, the result of new apatite formation. At day 7, the intensity of all the PO_4^{3-} peaks at the wave number both 566 and 1030 cm^{-1} increased, and CO_3^{2-} characteristic peaks at the wave number of 872 cm^{-1}were also detectable (Fig.7), which indicated that the apatite was formed was carbonate-apatite.[32,33] The mechanism of apatite formation on MBG is due to silicate dissolution and re-deposition of Ca and P ions, as well as the effect of specific mesopore structure.[12,16] One of the principal aims of the present study was to demonstrate controlled protein loading and release by controlling the density

of the apatite formed on the surface of MBG microspheres. Our results showed that as SBF soaking time increased, there was an increased apatite deposition, with a more pronounced crystalline structure, on the MBG microsphere surface, a clear indication that the apatite forming ability of MBG microspheres is a function of the soaking time in SBF.

Bovine serum albumin (BSA) loading efficiency of MBG microsphere with a function of apatite formation

TGA analysis showed there was an obvious weight loss for all the microspheres with increased temperature (Fig. 8). From room temperature to 200 ºC the weight loss was mainly due to the loss of water, whereas from 200 to 800 ºC the weight loss was mainly due to the burning out of the BSA. The BSA loading efficiency was calculated according to (weight loss)% in the 200-800ºC range. Table 2 shows the loading efficiency of BSA on MBG microspheres at 0, 1, 3 and 7 days soaking in SBF. Loading efficiencies of BSA improved significantly by co-precipitation with apatite compared to MBG microspheres without apatite, reaching 16.51% at day 7, more than 4 times than that of pure MBG microspheres. These results suggest that dissolving BSA in SBF solution, and co-precipitating with apatite onto MBG microspheres, is an efficient way to enhance the loading efficiency of BSA. Figure 10 shows a schematic model of how BSA is thought to be loaded into the MBG microspheres with respect to the incubation time in SBF. At all four time points, the BSA is trapped in the inner MBG microspheres within the loose structure and micropores, and binds with hydroxyl groups on the surface of the MBG particles by hydrogen bonds [16]. As the apatite accretes on the microspheres, the BSA binds to the MBG particles, as well as the apatite particles on the microspheres, by the chemical groups OH^-, PO_4^{3-} and CO_3^{2}, thereby enhancing the BSA loading ability. Our results showed that the loading capacity of BSA increased with increased soaking time

in SBF. The most likely explanation for this is that the increased density of apatite particles (shown in the section 2.3) allows binding of more BSA molecules; the BSA loading is therefore effectively controlled by the soaking time in the SBF solution. It is likely that this method can be used for controlled protein loading with other bioactive ceramics materials as well.

Controllable release kinetics of BSA from MBG microspheres

The chief finding from this study was that BSA release kinetics from MBG microspheres can be controlled by the accretion of an apatite layer on the surface of MBG microspheres. Figure 9 shows that MBG microspheres without an apatite layer had a burst release of BSA at the early stage, whereas MBG microspheres with an apatite layer had slower release kinetics. As the soaking time in SBF increased, there was a corresponding decrease in the BSA release kinetics (Fig.9). We also demonstrated a positive correlation between the length of time the microspheres were incubated in SBF and the amount of apatite formation, which to us suggested that the apatite itself played a role in the rate of BSA release. There are two likely explanations for this phenomenon: (i) that the apatite particles on the microsphere surface have greater specific surface area and therefore more chemical groups with which to bond BSA through molecular interactions, thereby decreasing the release rate of BSA; or (ii) that a denser apatite layer forms on the microsphere surface with increased soaking time in SBF, which may then retard the release of BSA from within the microspheres. The morphology of MBG microspheres after 28 days' release in PBS has been observed and shown in Figure 10. It is obvious that a layer of nano-apatite particles formed on the surface of MBG microspheres after release of BSA in PBS. Figure 11 shows a schematic model of how BSA capture is thought to accumulate as a function soaking time in SBF, and then subsequently released from the microspheres. Our results showed that the accumulative release of

BSA from the MBG-SBF-7d microspheres after 28 days in PBS, was no more than 8%, suggesting that most of BSA was trapped in, or by, the apatite layer. BSA can be incorporated into the lattice structure of the Ca-P minerals through the electrostatic interactions between BSA and the crystals[34], making it reasonable to speculate that the BSA was chemically bound to the apatite crystals rather than simply being adsorbed to the surface of the crystals. From our study, it is speculated that parts of BSA may embed in the internal pores (nano-pore channel) of MBG, which results in a sustained release even after 28 days of release, and the total release of BSA in pure MBG microspheres is no more than 40%. However, the apatite layer on the surface of MBG microspheres plays a key role to decrease the burst release of BSA, which indicates that BSA may form strong chemical bonding with apatite particles on the surface of MBG microspheres. Furthermore, the release kinetics of BSA at the early stage is much higher than that in the late stage, which indicates that the release behaviour of BSA in MBG system at early stage is mainly controlled by a diffusing way. However, the release kinetics in late stage is influenced by the degradation of the delivery system and re-precipitation of apatite particles (Figure 10). Our results indicate that controlling the density of apatite by varying the soaking time in SBF is a facile, yet useful way to control protein release.

Other investigators have confirmed that bone-like apatite layers possess a capability to enhance osteoblastic activity,[35,36] and previous work by our group has shown that apatite layers on the surface of bioactive ceramic bredigite ($Ca_7MgSi_4O_{16}$) scaffolds enhanced osteoblast spreading, proliferation and differentiation,[37] suggesting that the bone-like apatite layer formed on MBG microspheres may in fact enhance their cellular activity. In addition to showing that protein loading and release can be controlled by the apatite layer on MBG microspheres, we also showed that the Si containing ionic products from MBG microspheres can stimulate BMSCs proliferation. Therefore, compared with current microspheres, such as polymer and HAp microspheres, as bone filler

materials, the significant advantages of current MBG microspheres are that 1) they have a more controllable protein-delivery ability; 2) they have improved bioactivity (apatite-formation ability in SBF); 3) They are able to stimulate bone cell proliferation by releasing bioactive Si ions. Together these data lend support to MBG microspheres being a promising material for cell growth and controllable delivery of growth factor/drug, with a great potential for bone tissue regeneration application.

CONCLUSIONS

MBG microspheres with uniform morphology, large size and loose microstructure were successfully prepared by the method of combining alginate cross-linking $CaCl_2$ with heat treatment. These microspheres supported BMSC attachment and the Si containing ionic products from MBG microspheres stimulated cell proliferation. The BSA-loading efficiency of MBG was significantly enhanced by co-precipitating with apatite, and it appears that both loading efficiency and release kinetics of BSA can be controlled by controlling the density of the apatite formed on MBG microspheres by varying the soaking time in SBF. Altering the soaking time of bioactive MBG microspheres in SBF is therefore a facile and useful way to control the apatite-formation ability, and by so doing, to control BSA loading and release. These results suggest that MBG microspheres are a promising filling material for applications in bone regeneration by virtue of their controllable protein-delivery, as well as excellent bioactivity and degradation properties.

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REFERENCES

1. Barrias CC, Ribeiro CC, Lamghari M, Miranda CS, Barbosa MA. Proliferation, activity, and osteogenic differentiation of bone marrow stromal cells cultured on calcium titanium phosphate microspheres. J Biomed Mater Res A 2005;72(1):57-66.

2. Kang SW, Yang HS, Seo SW, Han DK, Kim BS. Apatite-coated poly(lactic-co-glycolic acid) microspheres as an injectable scaffold for bone tissue engineering. J Biomed Mater Res A 2008;85(3):747-756.

3. Malafaya PB, Santos TC, van Griensven M, Reis RL. Morphology, mechanical characterization and in vivo neo-vascularization of chitosan particle aggregated scaffolds architectures. Biomaterials 2008;29(29):3914-3926.

4. Luciani A, Coccoli V, Orsi S, Ambrosio L, Netti PA. PCL microspheres based functional scaffolds by bottom-up approach with predefined microstructural properties and release profiles. Biomaterials 2008;29(36):4800-4807.

5. Hsu FY, Chueh SC, Wang YJ. Microspheres of hydroxyapatite/reconstituted collagen as supports for osteoblast cell growth. Biomaterials 1999;20(20):1931-1936.

6. Li H, Chang J. Preparation, characterization and in vitro release of gentamicin from PHBV/wollastonite composite microspheres. J Control Release 2005;107(3):463-473.

7. Ribeiro CC, Barrias CC, Barbosa MA. Preparation and characterisation of calcium-phosphate porous microspheres with a uniform size for biomedical applications. J Mater Sci Mater Med 2006;17(5):455-463.

8. Santoni BG, Pluhar GE, Motta T, Wheeler DL. Hollow calcium phosphate microcarriers for bone regeneration: in vitro osteoproduction and ex vivo mechanical assessment. Biomed Mater Eng 2007;17(5):277-289.

9. Mateus AY, Barrias CC, Ribeiro C, Ferraz MP, Monteiro FJ. Comparative study of nanohydroxyapatite microspheres for medical applications. J Biomed Mater Res A 2008;86(2):483-493.

10. Tabata Y. Biomaterial technology for tissue engineering applications. J R Soc Interface 2009;6 Suppl 3:S311-324.

11. Hench LL. Bioceramics: from concept to clinic. J Am Ceram Soc 1991;74:1487-1510.

12. Yan X, Yu C, Zhou X, Tang J, Zhao D. Highly ordered mesoporous bioactive glasses with superior in vitro bone-forming bioactivities. Angew Chem Int Ed Engl 2004;43(44):5980-5984.

13. Vallet-Regi M, Balas F, Arcos D. Mesoporous materials for drug delivery. Angew Chem Int Ed Engl 2007;46(40):7548-7558.

14. Lopez-oriega A, Arcos D, Izquierdo-Barb I, Sakamoto Y, Terasaki O, Vallet-Regi M. Ordered mesoporous bioactive glasses for bone tissue regeneration. Chem Mater 2006;18:3137-3144.

15. Wu C, Ramaswamy Y, Zhu Y, Zheng R, Appleyard R, Howard A, Zreiqat H. The effect of mesoporous bioactive glass on the physiochemical, biological and drug-release properties of poly(DL-lactide-co-glycolide) films. Biomaterials 2009;30(12):2199-2208.

16. Xia W, Chang J. Well-ordered mesoporous bioactive glasses (MBG): a promising bioactive drug delivery system. J Control Release 2006;110(3):522-530.

17. Xia W, Chang J. Preparation, in vitro bioactivity and drug release property of well-ordered mesoporous 58S bioactive glass. J Non-Cryst Solids 2008;15:1338-1341.

18. Liu Y, Layrolle P, de Bruijn J, van Blitterswijk C, de Groot K. Biomimetic coprecipitation of calcium phosphate and bovine serum albumin on titanium alloy. J Biomed Mater Res 2001;57(3):327-335.

19. Barrere F, Snel MM, van Blitterswijk CA, de Groot K, Layrolle P. Nano-scale study of the nucleation and growth of calcium phosphate coating on titanium implants. Biomaterials 2004;25(14):2901-2910.
20. Du C, Schneider GB, Zaharias R, Abbott C, Seabold D, Stanford C, Moradian-Oldak J. Apatite/amelogenin coating on titanium promotes osteogenic gene expression. J Dent Res 2005;84(11):1070-1074.
21. Jongpaiboonkit L, Frankilin-Ford T, Murphy W. Mineral-coated polymer microspheres for controlled protein binding and release. Adv Mater 2009;21:1-4.
22. Zhu Y, Wu C, Ramaswamy Y, Kockrick E, Simon P, Kaskel S, Zreiqat H. Preparation, characterization and in vitro bioactivity of mesoporous bioactive glasses (MBGs) scaffolds for bone tissue engineering. Micropor Mesopor Mat 2008;112(1-3):494-503.
23. Mareddy S, Crawford R, Brooke G, Xiao Y. Clonal isolation and characterization of bone marrow stromal cells from patients with osteoarthritis. Tissue Eng 2007;13(4):819-829.
24. ISO/EN 10993-5 Biological evaluation of medical devices-part 5 tests for cytotoxicity, in vitro methods 82 tests on extracts:ISO/EN 10993-10995. Biological evaluation of medical devices-part 10995 tests for cytotoxicity, in vitro methods 10998.10992 tests on extracts.
25. Kokubo T, Takadama H. How useful is SBF in predicting in vivo bone bioactivity? Biomaterials 2006;27(15):2907-2915.
26. HE XM, Carter D. Atomic structure and chemistry of human serum albumin. Nature 1992;358:209-215.
27. Xynos ID, Edgar AJ, Buttery LD, Hench LL, Polak JM. Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce insulin-like growth factor II mRNA expression and protein synthesis. Biochem Biophys Res Commun 2000;276(2):461-465.
28. Gough JE, Jones JR, Hench LL. Nodule formation and mineralisation of human primary osteoblasts cultured on a porous bioactive glass scaffold. Biomaterials 2004;25(11):2039-2046.
29. Wu C, Chang J, Ni S, Wang J. In vitro bioactivity of akermanite ceramics. J Biomed Mater Res A 2006;76(1):73-80.
30. Sun H, Wu C, Dai K, Chang J, Tang T. Proliferation and osteoblastic differentiation of human bone marrow-derived stromal cells on akermanite-bioactive ceramics. Biomaterials 2006;27(33):5651-5657.
31. Huang Y, Jin X, Zhang X, Sun H, Tu J, Tang T, Chang J, Dai K. In vitro and in vivo evaluation of akermanite bioceramics for bone regeneration. Biomaterials 2009;30(28):5041-5048.
32. Liu X, Ding C, Chu PK. Mechanism of apatite formation on wollastonite coatings in simulated body fluids. Biomaterials 2004;25(10):1755-1761.
33. Wu C, Chang J. Degradation, bioactivity, and cytocompatibility of diopside, akermanite, and bredigite ceramics. J Biomed Mater Res B Appl Biomater 2007;83(1):153-160.
34. Wassell DT, Hall RC, Embery G. Adsorption of bovine serum albumin onto hydroxyapatite. Biomaterials 1995;16(9):697-702.
35. Olmo N, Martin AI, Salinas AJ, Turnay J, Vallet-Regi M, Lizarbe MA. Bioactive sol-gel glasses with and without a hydroxycarbonate apatite layer as substrates for osteoblast cell adhesion and proliferation. Biomaterials 2003;24(20):3383-3393.
36. Loty C, Sautier JM, Boulekbache H, Kokubo T, Kim HM, Forest N. In vitro bone formation on a bone-like apatite layer prepared by a biomimetic process on a bioactive glass-ceramic. J Biomed Mater Res 2000;49(4):423-434.
37. Wu C, Chang J, Zhai W, Ni S. A novel bioactive porous bredigite ($Ca_{7}MgSi_{4}O_{16}$) scaffold with biomimetic apatite layer for bone tissue engineering. J Mater Sci Mater Med 2007;18:857-864.

Figure captions

Figure 1. Optical pictures for MBG microsphere with uniform size (a), SEM pictures for single microsphere (b) and surface microstructures (c), and inner microstructure (d). EDS analysis shows that the ratio of Ca/P is 3.06 in (c).

Figure 2. Small angle XRD (a), TEM (b), and N2 adsorption-desorption isotherms and the corresponding pore size distributions (inset) (c) analysis for MBG microspheres.

Figure 3. The effect of ionic products from MBG microspheres on bone marrow stromal cell (BMSC) viability. The number of cell attachment at day 1 was similar in different MBG extract concentrations compared with the control and tissue culture plate. However, cell number was significantly increased in all groups containing MBG extract after three days in culture compared with the cell number in tissue culture plate, which indicated an enhanced effect of MBG extract on cell proliferation. The corresponding Ca and Si ion concentrations at different extract concentration were listed in Table 1.

Figure 4. BMSC attachment on MBG microspheres after culturing for 3 days. Fluorescent phalloidin (red) marking actin filaments in BMSCs, and nuclei with DAPI (blue).

Figure 5. SEM and EDS analysis of MBG microspheres soaked in SBF for 1 d (a), (d) and (g); 3d (b), (e) and (h); 7d (c), (f) and (i). The corresponding ratios of Ca/P are 2.52, 1.73 and 1.91. (d), (e) and (f) are higher magnification SEM.

Figure 6. Wide angle XRD analysis of MBG microspheres after soaking in SBF for 1, 3 and 7 days.

Figure 7. FTIR analysis of MBG microspheres after soaking in SBF for 1, 3 and 7 days.

Figure 8. TGA analysis of MBG microspheres loaded with BSA by SBF soaking for different time periods.

Figure 9. BSA release kinetics (accumulative release %) from MBG microspheres treated by SBF in

PBS at pH 7.4 for 28 days (a). Figure (b) is listed to show the difference of BSA release in the early stage (3 days).

Figure 10. Surface morphology of MBG microspheres after releasing of BSA in PBS for 28 days.

Figure 11. Schematic illustrations of BSA loading and release in four MBG microspheres. The left column is BSA loading and the right column is BSA release. With the increase of soaking time in SBF, more and more apatite particles deposited on the surface of MBG microspheres, which enhanced BSA loading efficiency and decreased BSA release kinetics.